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**Genomics, Proteomics, and  
Bioinformatics:**  
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## Calcyclin, a $\text{Ca}^{2+}$ Ion-binding Protein, Contributes to the Anabolic Effects of Simvastatin on Bone\*

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**In vitro** treatment with a pharmacological dose of simvastatin, a potent pro-drug of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, stimulates bone formation. In our study, simvastatin stimulated differentiation of osteoblasts remarkably in a dose-dependent manner, with minimal effect on proliferation. To identify the mediators of the anabolic effects of simvastatin on osteoblasts, we tried to identify and characterize simvastatin-induced proteins by using proteomic analysis. Calcyclin was significantly up-regulated by more than 10 times, and annexin I was also up-regulated by simvastatin. However, annexin III, vimentin, and tropomyosin were down-regulated. Up-regulated calcyclin mRNA by simvastatin was validated by reverse transcription in mouse calvarial cells. In confocal microscope analysis, green fluorescence protein-calcyclin fusion protein was ubiquitously observed in the of MC3T3-E1 cells transfected with green fluorescence protein-calcyclin cDNA containing plasmid and was quickly concentrated in the nucleus 20 min after simvastatin treatment. Overexpression of calcyclin cDNA stimulated both the proliferation and expression of alkaline phosphatase mRNA significantly, without exposure to simvastatin in MC3T3-E1 cells. However, both the rate of proliferation of the osteoblasts and the expression of alkaline phosphatase mRNA were suppressed significantly 1 day after treatment with the calcyclin-specific small interference RNA, and furthermore, simvastatin did not overcome this suppression in the small interference RNA-pretreated MC3T3-E1 cells. In conclusion, calcyclin is one of the candidate proteins that plays a role in osteoblastogenesis in response to simvastatin, although the precise functions of calcyclin in osteoblast remain to be verified.

Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing to an increased fracture risk (1, 2). The mainstay of therapy for osteoporosis still focuses on anti-resorptive agents (3). Anti-resorptive agents stabilize the bone remodeling by reducing the number and/or the activity of osteoclasts, thereby reducing the risk of fracture without increments of true bone mass (4). In contrast, anabolic agents such as parathyroid hormone (PTH),<sup>1</sup> statin, and fluoride directly

stimulate bone formation and increase bone mass (5, 6). Recently, the Food and Drug Administration approved PTH(1–34) as the first bone anabolic agent for severe osteoporosis. However, there are several undefined questions regarding the cortical porosity and the possibility of tumorigenesis induced by PTH(1–34). Therefore, the development of new anabolic agents is certainly mandatory (7–9).

Statin is a potent pro-drug of a hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor that is known to inhibit the conversion of hydroxy-3-methylglutaryl-coenzyme A to mevalonic acid, needed for hepatic cholesterol biosynthesis (6, 10). Mevalonic acid is a precursor not only of cholesterol but also of proteins such as geranylgeranyl pyrophosphate, which is important in the control of osteoclast-mediated bone resorption (11, 12). Previous studies have indicated that some lipophilic statins have a potent stimulatory effect on bone formation by inducing the expression of the bone morphogenetic protein (BMP)-2 (6, 10, 13). However, the detailed mechanisms of how statins promote bone formation have yet to be elucidated. Little is currently known about the intracellular statin-induced proteins, as well as the mediator proteins specific for the anabolic function of statins.

Calcyclin (S100A6), a small acidic protein that weighs about 10 kDa, belongs to the S100 calcium-binding protein family (14). These family members share a common S100 calcium-binding motif and are involved in several regulatory functions that include protein phosphorylation, some enzyme activities, the dynamics of cytoskeletal components, transcription factors, and  $\text{Ca}^{2+}$  homeostasis, and also cell proliferation and differentiation (15, 16). An interesting feature of the S100 proteins is that they are expressed by epithelial cells and fibroblasts in a cell-specific way (17). Calcyclin is also expressed by osteoblasts (MC3T3-E1 cells) and up-regulated markedly during osteoblast differentiation; however, its roles in bone physiology are unknown (18).

In the present study, we reassessed whether simvastatin regulates the proliferation and differentiation of osteoblast-like cells, and we also explored new protein profiles by proteomic techniques in response to simvastatin. Here, we found that calcyclin, the calcium-binding protein, is one of the simvastatin-induced proteins up-regulated by more than 10 times. Furthermore, overexpression of calcyclin by transient transfection stimulated both the proliferation and differentiation of MC3T3-E1 cells, as well as inhibiting those without simvastatin treatment through suppressing calcyclin by the siRNA. We

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<sup>1</sup> The abbreviations used are: PTH, parathyroid hormone; GFP, green

fluorescence protein; EGFP, enhanced GFP; siRNA, small interference RNA; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry;  $\alpha$ -MEM,  $\alpha$ -modification of Eagle's medium; RT, reverse transcription; ALP, alkaline phosphatase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline.

A

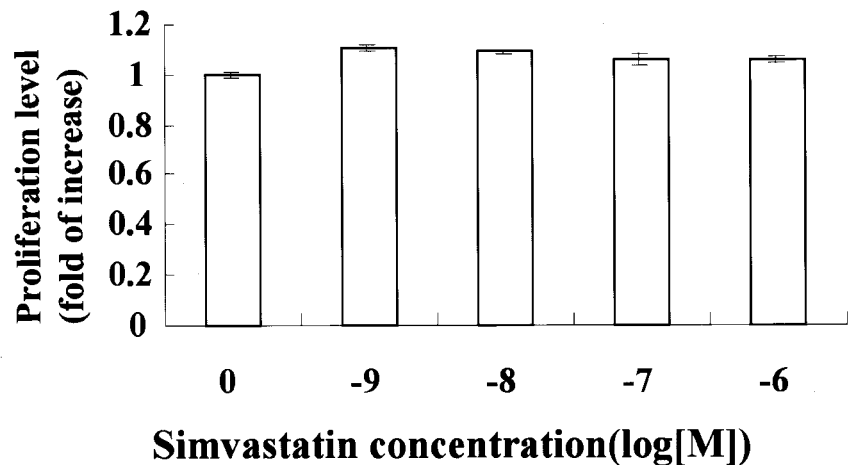
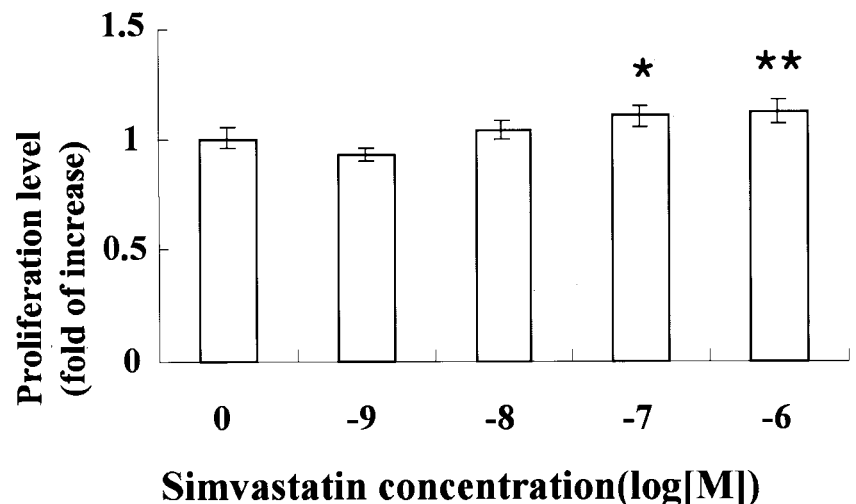


FIG. 1. The effect of short and long term exposure of simvastatin on proliferation of mouse calvarial cells. A, the short term effect of simvastatin on mouse calvarial cells was determined after 6 h of treatment. B, to study the long term effects of simvastatin, mouse calvarial cells were cultured for 8 days with intermittent simvastatin treatment (6 h/2 days) in various concentrations ( $10^{-9}$ – $10^{-6}$  M). After the incubation periods, the absorbance was determined by an enzyme-linked immunosorbent assay reader. The data represent the means of triplicate determination. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  simvastatin *versus* control.

B



propose that calcyclin could be one of the mediator molecules inducing the osteogenic functions of simvastatin.

#### EXPERIMENTAL PROCEDURES

**Cell Cultures**—Mouse calvariae were dissected aseptically from post-natal 1-day Institute of Cancer Research (ICR) mouse. Frontal and parietal bones were cleaned of loose soft connective tissue and submerged in an  $\alpha$ -modification of Eagle's medium ( $\alpha$ -MEM) (Invitrogen). Calvariae were digested at 37 °C for 10 min with shaking in an enzymatic solution containing 0.1% collagenase (Invitrogen) and 0.05% trypsin containing 0.53 mM EDTA (Invitrogen) in  $\alpha$ -MEM. This procedure was repeated to yield a total of five digests. The cells were collected by centrifugation at 1200 rpm for 5 min and were then resuspended in  $\alpha$ -MEM containing 10% fetal bovine serum and antibiotics (100 unit/ml penicillin and 100 mg/ml streptomycin; all from Invitrogen). The cells were grown to 70–80% confluence over the next 3–4 days at 37 °C in 5% CO<sub>2</sub> with humidification.

MC3T3-E1 cells were grown in  $\alpha$ -MEM with 10% fetal bovine serum. After the cells reached confluence, they were cultured in differentiation medium ( $\alpha$ -MEM containing 50  $\mu$ g/ml of phosphate ester of ascorbic acid (Sigma), 10 mM  $\beta$ -glycerophosphate (Sigma), and  $10^{-8}$  M dexamethasone (Sigma) for 5 days and then subjected to transfection.

**Cell Proliferation and Differentiation Assay**—Primary-cultured calvarial cells were cultured in a 96-well plate and stimulated for only 6 h with  $10^{-7}$  M simvastatin in serum-free  $\alpha$ -MEM. To assess the long term effects of simvastatin, the cells were cultured for 8 days being treated with simvastatin every other day for 6 h intermittently. Cell proliferation assay was performed using Cell Proliferation Reagent WST-1 (Roche Applied Science) following the manufacturer's instructions. For

the analysis of cell differentiation, total RNA was extracted from the cultured cells with a commercial RNeasy kit (Qiagen) following the manufacturer's instructions. The purity and amount of isolated RNA were assessed by spectrophotometric measurement at 260 and 280 nm. Total RNA (5  $\mu$ g) was reverse transcribed to cDNA at 42 °C for 50 min in a volume of 20  $\mu$ l containing the following reagents: 0.5 mM dNTP mixture; 10 mM dithiothreitol; 0.5 mg of oligo(dT), 1 $\times$  buffer (250 mM Tris, 375 mM KCl, and 15 mM MgCl<sub>2</sub>, pH 8.3), and 5 units of RNase H-free avian myeloblastosis virus reverse transcriptase (Promega). Then the reaction was terminated at 70 °C for 15 min. Aliquots of the cDNA were diluted from 1:1 to 1:100. RT-PCR analysis were done: 94 °C for 5 min, 94 °C for 45 s, 60 °C for 45 s, 72 °C for 35 s (repeated for 30 cycles), and 72 °C for 15 min. The following oligonucleotides were used for RT-PCR amplification: calcyclin (sequences of EcoRI restriction enzyme site are underlined), forward 5'-GAGAATTCAGTGATCAGT-CATGG-3', reverse 5'-CAGAATTCAGAGCTTCATTGTAG-3'; alkaline phosphatase (ALP), forward 5'-GGGACTGGTACTCGGATAACG-3', reverse 5'-CTGATATGCGATGTCCTTGCA 3'; osteocalcin, forward 5'-CGGCCCTGAGTCTGACAAA-3', reverse 5'-GCCGGAGTCTGTTC-CTCCTT-3'; Type I collagen, forward 5'-GAGGCATAAAGGGTCATC-GTGG-3', reverse 5'-CATTAGGCGCAGGAAGGTCAGC-3'; and  $\beta$ -actin, forward 5'-TTCAACACCCAGCCATGT-3', reverse 5'-TGTGGT-ACGACCAGAGGCATAC-3'.

**Two-dimensional Gel Electrophoresis and Gel Staining**—Protein samples of mouse calvarial cells used for two-dimensional gel electrophoresis analysis were obtained by single simvastatin stimulation for 6 h, and nonstimulated samples were used as a control. The samples were homogenized in a double volume of lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris, and 20 mM dithiothreitol). Then cell lysates were

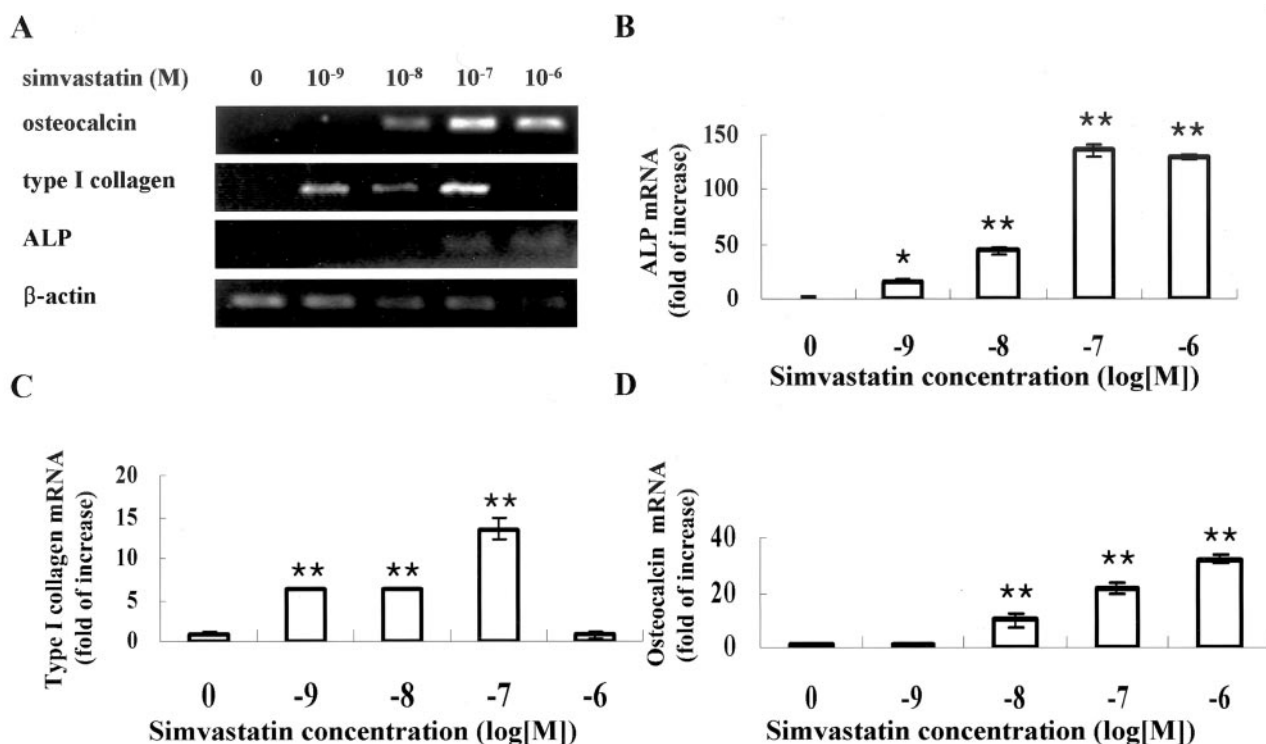


FIG. 2. The effect of simvastatin treatment on mRNA expression of osteoblast differentiation markers. A, ALP, type I collagen and osteocalcin mRNA expressions increased progressively and dose-dependently with simvastatin exposure over 6 h, whereas  $\beta$ -actin mRNA expression was not modified. B–D, the expression of each differentiation marker was investigated by gel electrophoresis and visualized by EtBr staining. All of the data illustrate the mean values of three different experiments and are normalized to those of  $\beta$ -actin. B, the mRNA expression level of ALP was significantly elevated in a dose-dependent manner. C, the mRNA expression level of type I collagen significantly increased dose-dependently but not at the  $10^{-6}$  M concentration. D, the mRNA expression level of osteocalcin increased significantly dose-dependently. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  simvastatin versus control.

centrifuged at 12,000 rpm for 15 min at 4 °C. The protein concentration was determined by the Bradford protein assay, and 100  $\mu$ g of proteins were used for the analysis. Commercial strips with a nonlinear immobilized pH 4–7 gradient (Amersham Biosciences) were used for isoelectric focusing. These strips were wetted with a rehydration buffer containing 8 M urea, 2% CHAPS, 10 mM dithiothreitol, and immobilized pH gradient buffer, pH 4–7 (Amersham Biosciences) for 12 h at 50 voltage. Isoelectric focusing was performed in the Multiphore II apparatus (Amersham Biosciences) for a total of 40,000 V-h. In the second dimension, the proteins were separated by size in a 7.5–17.5% T polyacrylamide gradient gel. Two slab gels were run using Protean II xi Cell (Bio-Rad) at a constant current of 20 mA for 14 h. After electrophoresis, the separated proteins were either silver-stained or Coomassie Brilliant Blue-stained. For silver staining, the gels were first fixed for 1 h in 40% ethanol, 5% acetic acid and then washed with 50 ml of double-distilled water followed by a second fixing in 5% ethanol, 5% acetic acid. The gels were washed with 50 ml of double-distilled water and incubated with a solution of 0.02% sodium thiosulfate (Sigma) for 30 min at room temperature. The gels were washed with  $2 \times 10$  ml of double-distilled water and incubated for 90 min at 4 °C with 0.1% silver nitrate solution that was precooled to 4 °C. The gels were rinsed with 50 ml of double-distilled water, and the proteins were visualized using a developing solution containing 0.5% formaldehyde, 2%  $\text{KCO}_4$  until the proteins were visible. Acetic acid solution (1%) was used to stop the reaction. For matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS), the gels were stained with Coomassie Blue G-250 (0.25%) in 50% methanol, acetic acid overnight at room temperature. Coomassie Blue-stained gels were destained with 40% methanol, 12% acetic acid until the background was clear.

**Image Analysis Using PDQuest Software and MALDI-TOF-MS**—Qualitative analysis of digitized images was carried out using PDQuest two-dimensional analysis software (Bio-Rad). The samples were loaded onto the target plate using the three layers method (19). 0.6  $\mu$ l of matrix solution (10 mg/ml sinapinic acid in 60% acetonitrile, 40% water, 0.1% trifluoroacetic acid, prepared fresh every day) were loaded onto the sample plates and left to dry at room temperature. One  $\mu$ l of sample solution was deposited on the matrix layer and left to dry. The spot was then covered with 0.6  $\mu$ l of the matrix solution. The mass spectra were

recorded using a ToF-Spec two-dimensional gel electrophoresis MALDI-TOF instrument (Micromass, Manchester, UK), equipped with a pulsed nitrogen laser (337 nm; pulse width, 4 ns), and operated in delayed extraction linear mode, with an acceleration voltage of 22.5 kV.

**pcDNA-cacy and pEGFP-cacy Construction and Transfection**—Full-length calcyclin cDNA amplified by RT-PCR as described above was sequenced and digested with EcoRI restriction enzyme and purified by agarose gel electrophoresis. The resulting restriction fragment containing the entire calcyclin coding region was ligated into the vector pcDNA 3.0 (Invitrogen) and pEGFP-N1 (Clontech). MC3T3-E1 cells were maintained in  $\alpha$ -MEM supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin sulfate, 100 units/ml penicillin G, and 250 mg/ml amphotericin B. MC3T3-E1 cells were transfected with pcDNA-cacy and pEGFP-cacy construct using LipofectAMINE reagent (Invitrogen). Briefly, once the MC3T3-E1 cells reached about 60–70% confluence, they were washed once with  $\alpha$ -MEM (serum-free; Invitrogen) and a mixture of 1  $\mu$ g of plasmid in a total volume of 1 ml/well in a 6-well plate. Transfection was allowed to proceed for 6 h and followed by the removal of the transfection medium and the addition of 2 ml  $\alpha$ -MEM containing 10% fetal bovine serum. The assays were performed the day after transfection.

**Confocal Microscopic Analysis**—For the calcyclin translocation assay, MC3T3-E1 cells were transfected with pEGFP-cacy plasmid. After various treatments,  $10^{-7}$  M simvastatin,  $10^{-7}$  M PTH(1–34) and co-incubation with both simvastatin and PTH for 20 min, pEGFP-cacy transfected cells were washed twice with PBS and fixed in 3% formaldehyde (in PBS) at room temperature for 20 min and then rinsed thoroughly with PBS. Confocal microscopic experiment was performed on a Zeiss LSM-510 laser scanning microscopy by using a Zeiss 100 $\times$  oil immersion lens. Fluorescent signals were collected by using Zeiss LSM software in the line-switching mode with dual excitation (488 nm, 568 nm) and emission (515–540 nm, 590–610 nm) filter sets. For the nuclear staining, Hoechst stock solution (final concentration, 0.12  $\mu$ g/ml) was added and incubated for 15 min at room temperature. After rinsing the cells with PBS for five times, the confocal images were visualized with a light source at 340–350 nm.

**siRNA Construction**—General guidelines for designing siRNA oligonucleotides are available (20) that require avoiding regions of mRNA



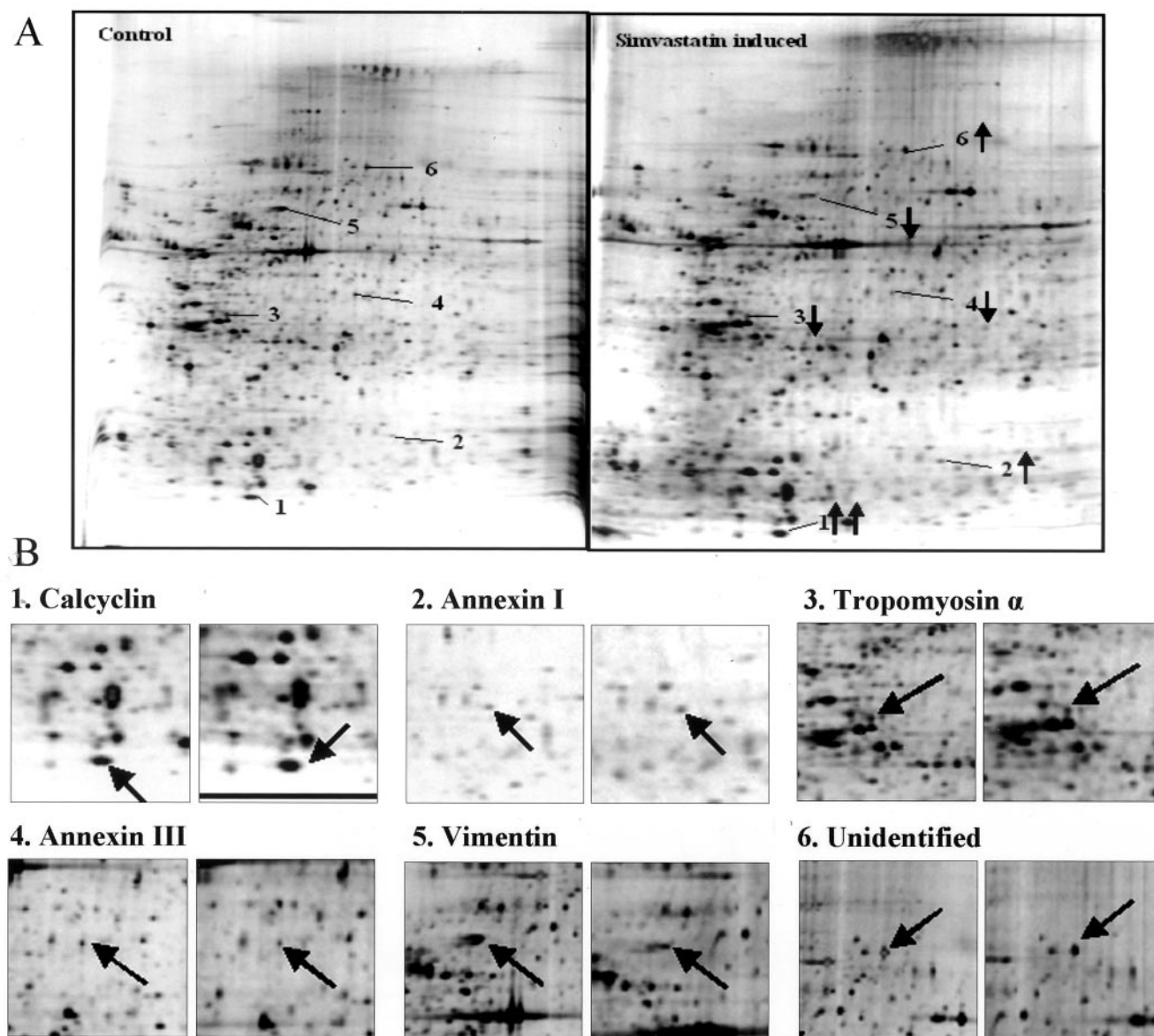


FIG. 3. Two-dimensional gel protein profiles after simvastatin treatment for 6 h in mouse calvarial cells. A, black circles indicate proteins whose expression was found to be lower or higher in simvastatin-induced culture relative to control. The horizontal axis of the gels is the isoelectric focusing dimension, which stretches from pH 4 (left panels) to pH 7 (right panels). The vertical axis is the polyacrylamide gel dimension, which stretches from about 8 kDa (bottom panels) to about 80 kDa (top panels).  $\uparrow$ , 4-fold increase;  $\downarrow$ , 4-fold decrease;  $\uparrow\uparrow$ , 10-fold increase. B, expanded images of each of the proteins indicated by the same numbers in A showing altered expression upon stimulation with simvastatin for 6 h.

such as 5'- and 3'-untranslated regions and regions within 75 bases of a start codon and sequences having a GC content of >70% or <30%. An appropriate siRNA sequence within the target calcyclin mRNA sequence was chosen according to the manufacturer's software provided by Ambion (Austin, TX). 5'-AAGGAAGGAGCTGAAGGAGTTG-3' and 5'-AAACTTCCAGGAGTATGTCGC-3' siRNA oligonucleotides were synthesized using the siRNA Construction kit (Ambion) following the manufacturer's instructions. Effective RNA interference probes and their effective concentration were determined based on their ability to inactivate cognate sequences. Calcyclin and the siRNA effect were monitored quantitatively using gel analysis. MC3T3-E1 cells were plated onto 6-well plates for RNA preparation and 96-well plates for the proliferation assay and then incubated for 24 h. The cells were then transfected with 10 nM siRNA using the LipofectAMINE reagent according to the manufacturer's instructions.

**Statistical Analysis**—All of the data were expressed as the means and standard deviation. SPSS 10.0 software (Chicago, IL) was used for the statistical analysis, and Student's *t* test was used for the comparison. The relationships were considered statistically significant when the *p* value was less than 0.05.

## RESULTS

**The Effect of Simvastatin on the Proliferation and Differentiation of Osteoblasts**—To examine the effect of simvastatin on both the proliferation and differentiation of osteoblasts, mouse calvarial cells were stimulated with simvastatin single treatment for 6 h. For the study of the long term effects of simvastatin, mouse calvarial cells were cultured for 8 days with intermittent simvastatin treatment (6 h/2 days) in various concentrations ( $10^{-9}$ – $10^{-6}$  M). Simvastatin was treated intermittently because it has been reported that statins exert a number of effects that include stabilizing cells and inhibiting cell proliferation (21, 22). Short term treatment of simvastatin was not particularly effective on osteoblastic proliferation (Fig. 1A) and exhibited a tendency to increase slightly for a long term effect (Fig. 1B). The proliferation was increased dose-dependently during simvastatin exposure periods and reached a maximum at  $10^{-6}$  M (Fig. 1B).

As shown in Fig. 2, incubation with  $10^{-7}$  M simvastatin for 6 h enhanced the expression of ALP, type I collagen and osteocalcin to a statistically significant extent, compared with that in the unstimulated conditions in cultured mouse calvarial cells. Because this increase was mostly dose-dependent ( $10^{-9}$ – $10^{-6}$  M), the following experiments were performed with stimulation by a final  $10^{-7}$  M of simvastatin for 6 h, unless otherwise indicated. Quiescent mouse osteoblasts expressed a low level of ALP mRNA, and this mRNA expression was significantly enhanced and saturated by  $10^{-7}$  M simvastatin treatment by more than 100-fold (Fig. 2B). Similar to the case of ALP, the increase in type I collagen expression was significant at a concentration of  $10^{-7}$  M and, interestingly, the expression dropped to the basal level at the concentration of  $10^{-6}$  M (Fig. 2C). In the case of osteocalcin, the mRNA expression increased dose-dependently and reached a maximum level at a concentration of  $10^{-6}$  M (~30-fold; Fig. 2D).

**Induction of Calcyclin Protein after Simvastatin Treatment in Mouse Calvarial Cells**—Fig. 3 shows an example of a silver-stained two-dimensional gel electrophoresis images aimed at determining any change after 6 h of treatment with simvas-

tin, and these images were analyzed by PDQuest software (described above). Six spots that showed expressional changes more than four times were obtained selectively from this analysis and characterized by MALDI-TOF-MS (Table I). Calcyclin protein, a member of the S100 calcium-binding protein family, showed a 10-fold increase after 6 h of simvastatin treatment. Annexin I and an unidentified spot also increased by more than four times. However, the others, which included tropomyosin, annexin III, and vimentin, showed a 4-fold decrease.

**The Effects of Simvastatin on mRNA Expression of Calcyclin**—To confirm the effect of simvastatin on the expression of calcyclin at the mRNA level, RT-PCR was performed with RNA obtained from mouse calvarial cells that were either untreated or treated for 6 h with simvastatin. Fig. 4A illustrates the mean values of three different experiments and shows that calcyclin mRNA increased progressively with the increasing time of simvastatin exposure. Calcyclin mRNA induction significantly increased as quickly as 30 min after the simvastatin addition and showed a maximal effect after 1 h (about 60% increase), and thereafter, the calcyclin mRNA expression returned to the control level by 24 h. Fig. 4B shows an example of RT-PCR aimed at determining the effective simvastatin concentration of calcyclin mRNA induction. The expression of calcyclin reached almost the maximal level at a concentration of  $10^{-9}$  M and maintained this level to the  $10^{-6}$  M concentration.

**The Distribution of Calcyclin after Simvastatin Treatment**—To determine the cellular localization of calcyclin following simvastatin stimulation, calcyclin was fused into enhanced green fluorescence protein (EGFP), and the fusion protein was transiently expressed in MC3T3-E1 cells. As shown in Fig. 5, the fluorescence distributions of the calcyclin were expressed ubiquitously through the unstimulated MC3T3-E1 cells and realized by confocal laser scanning microscope. Twenty min-

TABLE I  
Identities of the selective simvastatin-induced proteins characterized by MALDI-TOF-MS

Protein	pI	Molecular mass	Expression level
		<i>kDa</i>	
1 Calcyclin	5.40	10.5	10-fold increase
2 Annexin I	4.71	39.0	4-fold increase
3 Tropomyosin- $\alpha$	5.96	32.7	4-fold decrease
4 Annexin III	5.06	36.5	4-fold decrease
5 Vimentin		53.7	4-fold decrease
6 Unidentified			4-fold increase

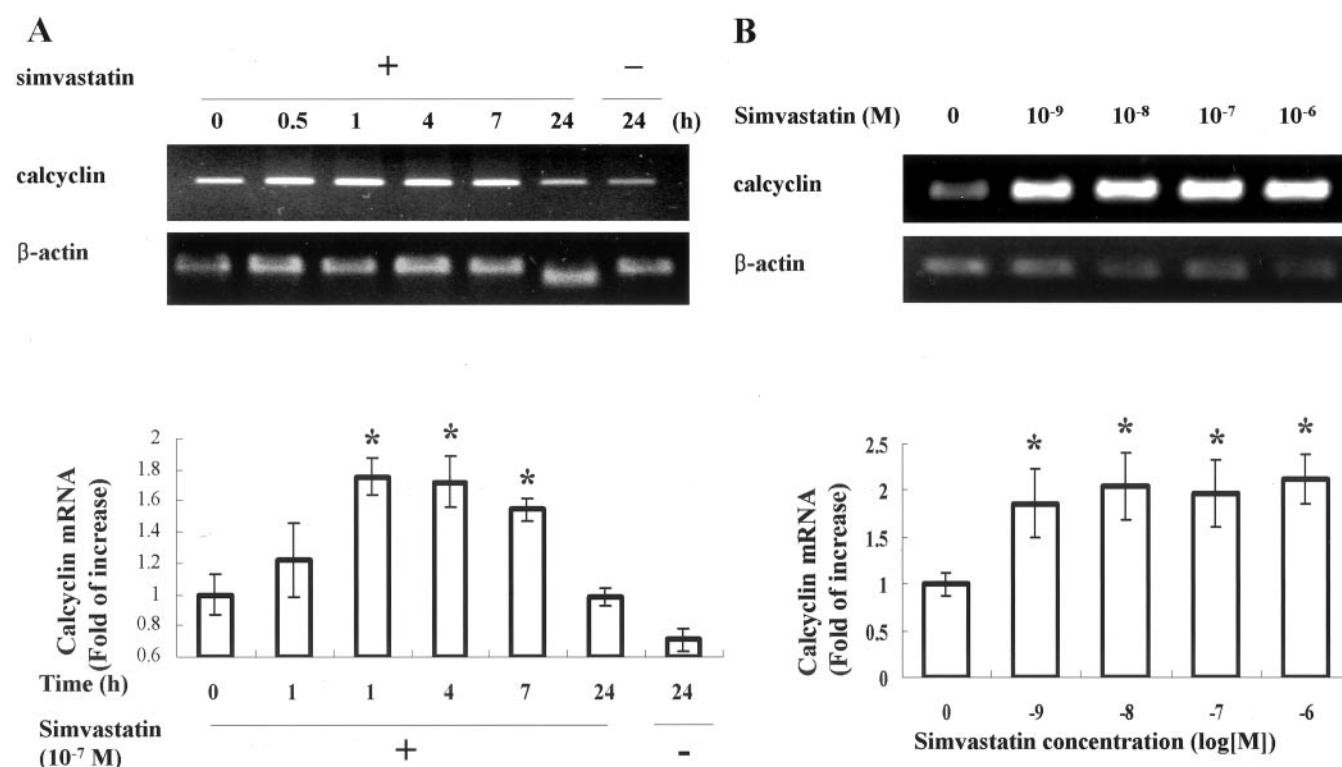
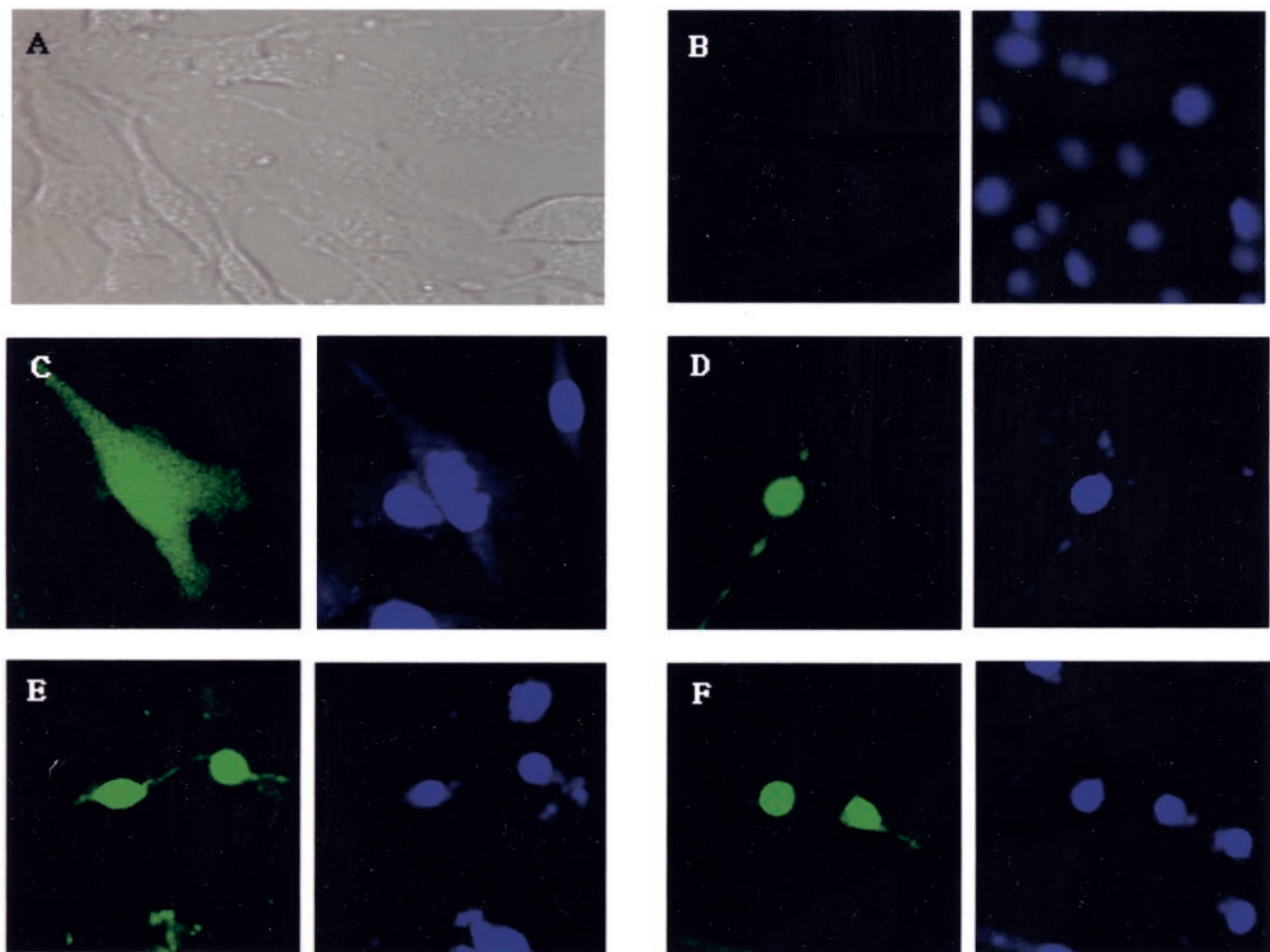


FIG. 4. Time- and dose-dependent induction of calcyclin mRNA expression after simvastatin treatment in mouse calvarial cells. A, the effect of treatment of mouse calvarial cells with simvastatin  $10^{-7}$  M for different time intervals was studied by RT-PCR. Calcyclin appeared as a single band at about 310 bp, and  $\beta$ -actin was used as an internal standard. Calcyclin mRNA expression was significantly increased by simvastatin after 1 h and then continued to increase for 7 h. Thereafter, it returned to the control value at 24 h. B, the effect of treatment of mouse calvarial cells with simvastatin at various doses was also studied. Calcyclin mRNA expression was saturated even at the lowest  $10^{-9}$  M concentration at 6 h after simvastatin treatment and showed similar values at  $10^{-8}$ ,  $10^{-7}$ , and  $10^{-6}$  M. \*,  $p < 0.05$ , simvastatin versus control.



**FIG. 5. Localization of calcyclin by confocal microscope in MC3T3-E1 cells.** Representative fluorescence images in MC3T3-E1 cells after various treatments,  $10^{-7}$  M simvastatin and  $10^{-7}$  M PTH(1–34), and co-incubation with both simvastatin and PTH for 20 min. For the nuclear staining, Hoechst stock solution (final concentration, 0.12  $\mu$ g/ml) was added and incubated for 15 min at room temperature. After rinsing the cells with PBS, confocal images were visualized with a light source at 340–350 nm on a Zeiss LSM-510 laser scanning microscopy by using a Zeiss 100 $\times$  oil immersion lens. **A**, optical image of MC3T3-E1 cells no transgene expressed. **B**, *left*, no GFP signal in the absence of pEGFP-cacy plasmid under confocal microscopy; *right*, Hoechst staining of the same cells showing the nuclei. **C**, in the presence of GFP-cacy fusion protein, a clear signal was evidenced in the cytoplasm, as well as the nuclei of the cells (in green). **D**, GFP-cacy fusion protein was quickly concentrated to the nucleus as quickly as 20 min after simvastatin treatment. **E** and **F**, a potent anabolic hormone, PTH(1–34), gave a similar effect on calcyclin localization, as well as following co-treatment with simvastatin. The nuclei were stained in blue.

utes after stimulation with simvastatin, the cellular EGFP-cacy was quickly localized to the nucleus. In these experiments, the nucleus was stained with Hoechst 33258. PTH(1–34) alone or a co-treatment of PTH(1–34) with simvastatin also revealed the similar effects on GFP-cacy localization.

**The Effects of Transient Transfection of pcDNA-cacy Plasmid on Proliferation and ALP mRNA Expression**—To examine the *in vitro* osteogenic effect of calcyclin in terms of proliferation and differentiation, we transiently transfected pcDNA-cacy in MC3T3-E1 cells. By comparison, calcyclin overexpressed cells showed significantly increased proliferation (Fig. 6A). The expression of ALP mRNA, which is an early osteoblast differentiation marker, increased significantly the day after the transfection, and the effects were normalized to  $\beta$ -actin mRNA expression (Fig. 6B). This might show the osteogenic potential of calcyclin in osteoblast *in vitro*. As for the mRNA expression of osteocalcin and type I collagen, there was no significant difference after pcDNA-cacy introduction (data not shown).

**The Effects of Complementary Double-stranded RNA against Calcyclin on Proliferation and ALP mRNA Expression in MC3T3-E1 Cells**—To determine whether the increased ALP mRNA and increased proliferation after pcDNA-cacy introduction were truly calcyclin-specific, RNA interference was per-

formed. Two 21-mer siRNAs with 19 complementary nucleotides were synthesized, and a more appropriate template sequence for calcyclin specific-siRNA was 5'-AGGAAGGAGCT-GAAGGAGTTG-3', which was named siCal (see "Experimental Procedures"). After siCal introduction to MC3T3-E1 cells, the expression of calcyclin mRNA was suppressed by  $\sim$ 90%, and simvastatin treatment did not alter the reduced calcyclin expression (Fig. 7). Entry of siCal performed by transient transfection using LipofectAMINE reduced the proliferation level by  $\sim$ 20% (Fig. 8A). The ALP mRNA expression level was tested and showed very similar but more drastic results (Fig. 8B). Interestingly, simvastatin induction could not alter the effects of siCal in either case (Fig. 8).

#### DISCUSSION

Recently, many experimental observations revealed that simvastatins, potent pro-drugs of a hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, stimulated bone formation. When mouse calvariae were incubated with simvastatin, greater numbers of osteoblasts and a 30–50% increase in adjacent bone width was noted (6). Subsequent epidemiological studies have suggested that treatment with simvastatin for hyperlipidemia may be associated with increased bone mineral



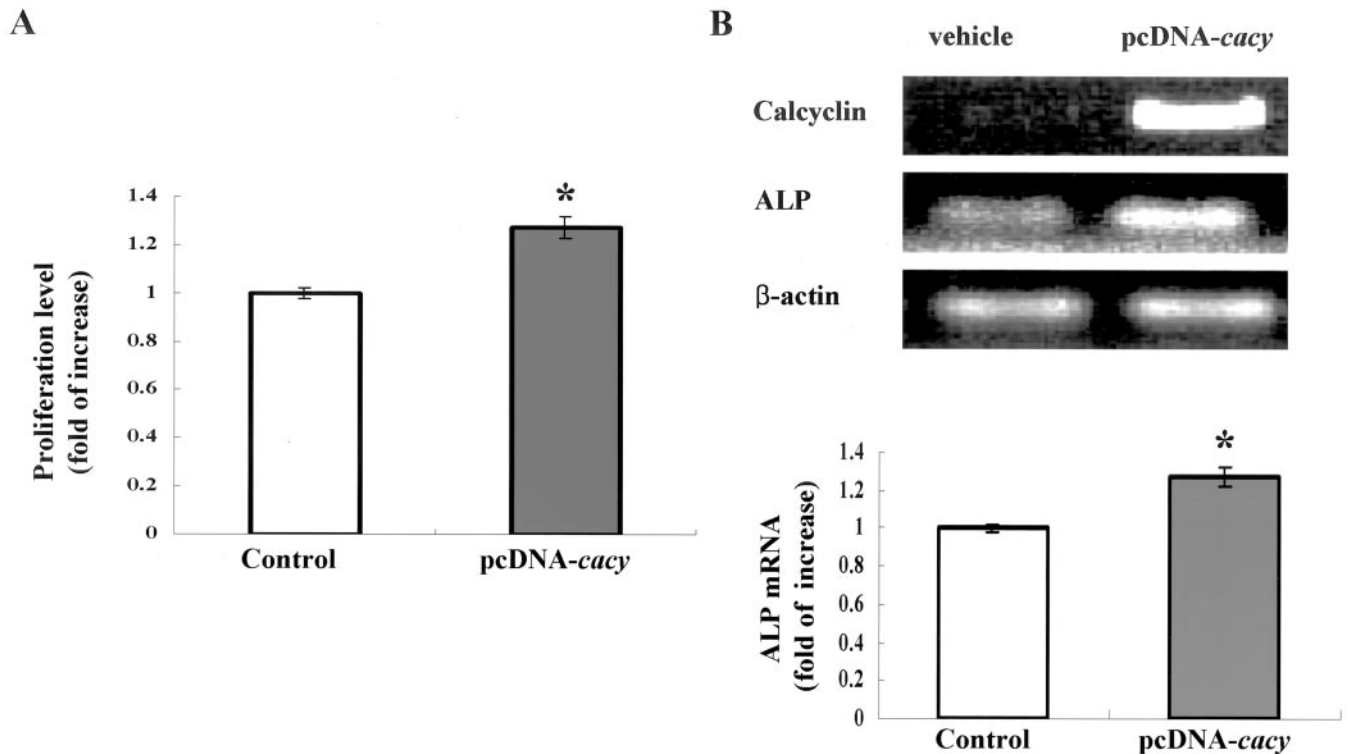


FIG. 6. **The effect of pcDNA-cacy transfection on proliferation and ALP mRNA expression.** A, pcDNA-cacy plasmid was introduced into MC3T3-E1 using LipofectAMINE, and the proliferation level was determined using WST-1 reagent the day after transfection. Proliferation of MC3T3-E1 cells was significantly increased by pcDNA-cacy introduction. B, RNA was harvested from MC3T3-E1 cells the day after transfection with vehicle only (pcDNA 3.0) or pcDNA-cacy and then screened by RT-PCR for ALP expression. ALP mRNA expression was significantly increased by pcDNA-cacy introduction. The data represent the means of triplicate determination. \*,  $p < 0.01$  pcDNA-cacy versus vehicle.

density and reduced fracture risk in humans, even though there are some controversies (23). The effects of simvastatin have been suggested to be mediated through the induction of potent bone-forming growth factors, BMPs. NO production through statin-induced up-regulated endothelial nitric-oxide synthase also plays some key role in regulating osteoblast activity and bone formation (24, 25). However, the action mechanisms of simvastatin for inducing pleiotropic effects on bone have not been well documented yet.

In this study, there was little significant difference on the degree of proliferation in the simvastatin single-treated mouse calvarial cells, if any, although a slight, dose-dependent increase of the proliferation occurred over time (8 days). However, simvastatin significantly increased the transcriptional level of osteoblast differentiation markers (Fig. 2). Following the induction of simvastatin for 6 h, the mRNA expression of alkaline phosphatase, type I collagen, and osteocalcin, were remarkably enhanced, however, with slightly different aspects in each. In the type I collagen mRNA, the expression level dropped dramatically to the basal level at the highest simvastatin concentration. In the previous studies, simvastatin and subsequently other lipophilic statins such as lovastatin, mevastatin, and fluvastatin also resulted in a 2–3-fold increase in new bone formation and osteoblast cell numbers at all stages of differentiation (6). Meanwhile, other bone growth factors, such as transforming growth factor and fibroblast growth factors were found to stimulate osteoblast proliferation but inhibit osteoblast differentiation (24, 25).

To identify some key molecules with roles in the anabolic effects of simvastatin, proteomics analysis was performed. In cultured mouse calvarial cells, calcyclin, annexin I and III, vimentin, and tropomyosin- $\alpha$  were significantly up- or down-regulated by simvastatin (Fig. 3). Among the proteins induced, the most strikingly increased protein was calcyclin (S100A6),

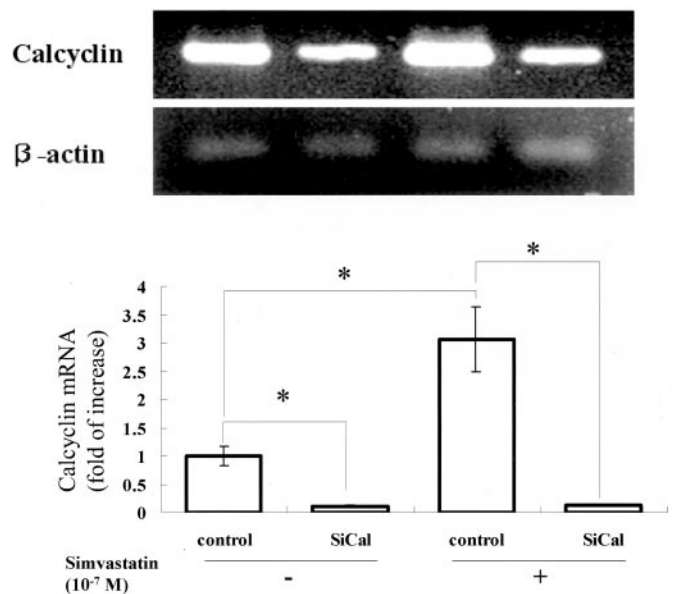


FIG. 7. **Calcyclin mRNA in MC3T3-E1.** Results of gel analysis of RT-PCR. Each bar represents the mean and standards of error for three separate experiments of calcyclin densitometry values normalized to  $\beta$ -actin. Subscripts (control and siCal-introduced) showed significantly different values. \*,  $p < 0.01$ . Calcyclin mRNA expression was significantly decreased by siCal and was not altered by simvastatin treatment.

which is a 10.5-kDa protein, belonging to the family of calcium-binding proteins. Calcium-binding proteins are divided into two groups; the first group is constituted of annexin, and the second group is constituted of EF hand proteins as calmodulin (15, 16, 26). Calcyclin (S100A6), which belongs to the second group, was first identified as a cellular cycle-dependent protein



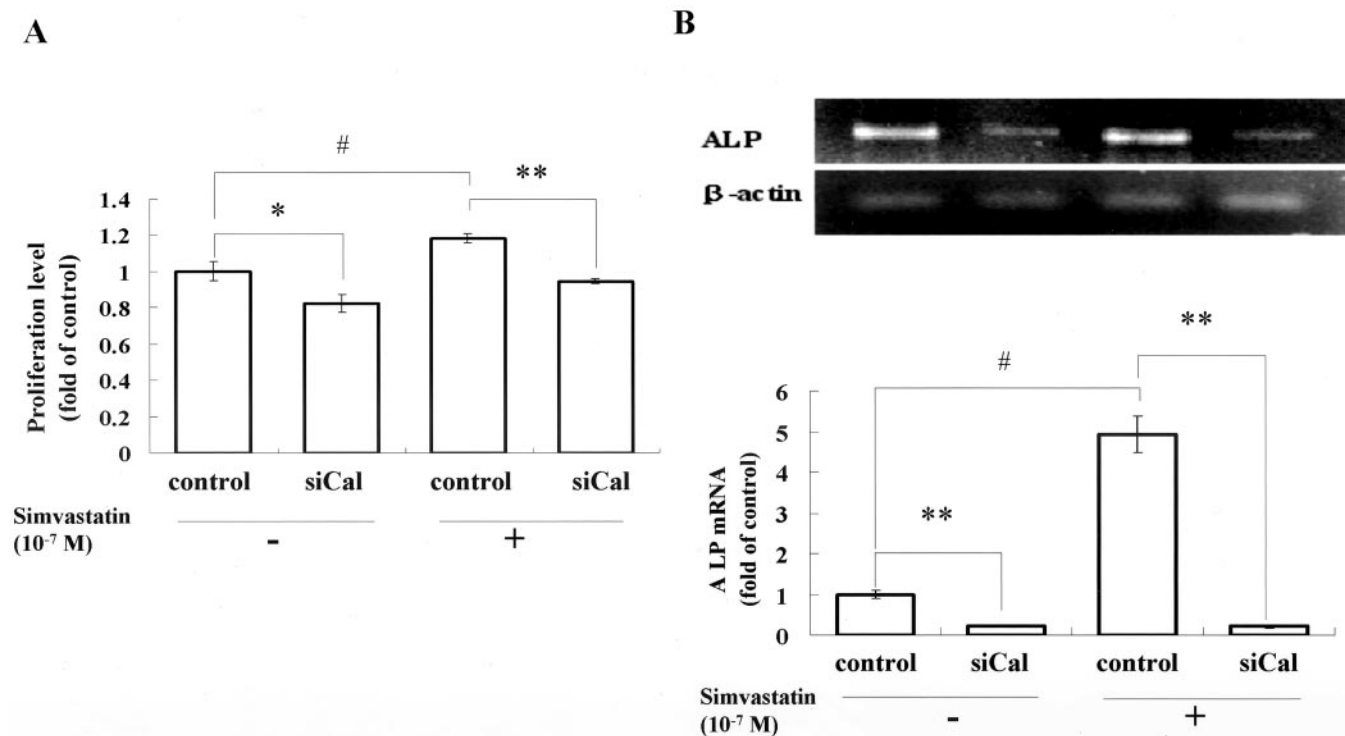


FIG. 8. The effects of siCal on proliferation and ALP mRNA expression in MC3T3-E1 cells. A, siCal had obvious effects in decreasing the MC3T3-E1 cell proliferation ( $p < 0.01$ ) just 1 day after its introduction. There was no significant cellular proliferation in calcyclin-suppressed cells treated with  $10^{-7}$  M simvastatin for 6 h. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus control; #,  $p < 0.05$  versus simvastatin-treated control. B, in RT-PCR analysis after siCal introduction, there was a marked decrease in ALP mRNA expression ( $p < 0.01$ ). Simvastatin did not alter the ALP mRNA expression when treated to a final concentration of  $10^{-7}$  M for 6 h, compared with the negative control (this is also siCal introduced). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus control; #,  $p < 0.05$  versus simvastatin-treated control.

highly induced by growth conditions (26). Furthermore, the expression of S100 in osteoblastic cells was verified in various types of osteosarcomas, in which immunohistochemical staining for S100 was used to analyze the proliferating cells in osteosarcomas, and the main proliferating cells to be stained were found to be mature osteoblast-like cells (27, 28). The existence of the S100 family in osteoblasts were confirmed in subsequent studies (18, 29).

S100 family members share a common S100 calcium-binding motif and have several regulatory functions in, not only protein phosphorylation, some enzyme activities, the dynamics of cytoskeletal components, transcription factors, and  $\text{Ca}^{2+}$  homeostasis but also the cell proliferation and differentiation (15, 16). S100 protein family has been known to exhibit an inhibitory role on protein phosphorylation and target intracellular proteins such as vimentin and annexin I and III (30–32). S100A4, one of the S100 family members expressed by osteoblastic cells, is a novel negative regulator of matrix mineralization that most likely acts by modulating the process of osteoblast differentiation (29). Meanwhile, calcyclin, S100A6, interacts with tropomyosin- $\alpha$  at the intracellular level (16). In this study, tropomyosin- $\alpha$ , a type of cytoskeleton, was down-regulated by simvastatin concurrent with the up-regulation of calcyclin. However, its precise role in osteoblasts remains unknown. Therefore, we focused our experimental efforts on calcyclin, which seemed to be the key protein regulated by simvastatin.

The mRNA expression of calcyclin was rapidly induced by simvastatin only 30 min following treatment (Fig. 4A), and the mRNA expression decreased rapidly to the control level 1 day after simvastatin induction (Fig. 4A). Because the mRNA expression of calcyclin was very sensitive to even the lowest dosage ( $10^{-9}$  M) of simvastatin, the effect of simvastatin seemed to be a quite strong and effective inducer of the calcyclin (Fig. 4B). Furthermore, simvastatin also exerted translocating effect

on calcyclin quickly after the exposure (Fig. 5). Calcyclin was localized ubiquitously throughout the cells, including the cytoplasmic compartment, in basal conditions, and it was concentrated quickly to the nucleus after 20 min of treatment with simvastatin. Meanwhile, parathyroid hormone, a potent anabolic hormone on bone, also induced the nuclear translocation of calcyclin in MC3T3E-1 cells. This fact may suggest that nuclear transferred calcyclin may turn on or off some important genes related to the anabolic effects on osteoblasts induced by either simvastatin or PTH(1–34). Recently, calcyclin was found to mediate the serum-responsive element activation by an osteoblastic extracellular cation-sensing mechanism in MC3T3-E1 cells (33).

To clarify the role of calcyclin on the anabolic effects of simvastatin on bone, calcyclin was overexpressed in MC3T3-E1 cells without treatment with simvastatin. Interestingly, both the rate of proliferation and the expression of alkaline phosphatase mRNA were elevated significantly 1 day after transfection (Fig. 6). Expression of calcyclin was abolished by up to almost 90% by treatment with siRNA against calcyclin (Fig. 7), and both the rate of proliferation and the expression of alkaline phosphatase mRNA were decreased significantly 1 day after the transfection with the siRNA (Fig. 8). Interestingly, simvastatin induction had no effect on the proliferation and ALP mRNA expression in calcyclin-suppressed MC3T3-E1 cells by siCal. These results provide strong evidence that calcyclin plays some important roles as a mediator of simvastatin-induced anabolic effects on bone.

The precise mechanism of how calcyclin has a biological effect on bone physiology and on osteoblast differentiation remains unclear. But, according to a recent report, S100A6 and other S100 proteins, through their interaction with ubiquitin ligases, can regulate the ubiquitination of  $\beta$ -catenin *in vivo* and thus participate in the process of tumor development and pro-

gression (34). Activation of endogenous  $\beta$ -catenin signaling in the canonical pathway of wnt induces the expression of alkaline phosphatase and subsequent osteoblast differentiation and, further, participates in BMP-2-mediated signal transduction (35, 36). Therefore, we speculate that in simvastatin-mediated osteoblast differentiation, calyculin, elevated by simvastatin treatment, causes defects in the ubiquitination of  $\beta$ -catenin and its degradation and thus can regulate the osteoblast differentiation. Another potential explanation might be that calyculin plays a role as a messenger for the induction of BMP-2 or NO synthase by statin. However, to provide clear evidence to these novel issues, there needs to be further investigation.

In conclusion, calyculin (S100A6) was identified as a new simvastatin-induced protein and revealed to play important roles in an anabolic effect of simvastatin on bone. Further clarification of calyculin function in osteoblast may discover novel targets for stimulating bone formation.

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